

University of Groningen

**Kinetic Properties of a Phosphate-Bond-Driven Glutamate-Glutamine Transport System in *Streptococcus lactis* and *Streptococcus cremoris***

POOLMAN, B; SMID, EJ; KONINGS, WN

*Published in:*  
Journal of Bacteriology

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
1987

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

POOLMAN, B., SMID, EJ., & KONINGS, WN. (1987). Kinetic Properties of a Phosphate-Bond-Driven Glutamate-Glutamine Transport System in *Streptococcus lactis* and *Streptococcus cremoris*. *Journal of Bacteriology*, 169(6), 2755-2761.

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

## Kinetic Properties of a Phosphate-Bond-Driven Glutamate-Glutamine Transport System in *Streptococcus lactis* and *Streptococcus cremoris*

BERT POOLMAN, EDDY J. SMID, AND WIL N. KONINGS\*

Department of Microbiology, University of Groningen, 9751 NN Haren, The Netherlands

Received 24 October 1986/Accepted 26 February 1987

In *Streptococcus lactis* ML3 and *Streptococcus cremoris* Wg2 the uptake of glutamate and glutamine is mediated by the same transport system, which has a 30-fold higher affinity for glutamine than for glutamate at pH 6.0. The apparent affinity constant for transport ( $K_T$ ) of glutamine is  $2.5 \pm 0.3 \mu\text{M}$ , independent of the extracellular pH. The  $K_T$ s for glutamate uptake are 3.5, 11.2, 77, and  $1200 \mu\text{M}$  at pH 4.0, 5.1, 6.0, and 7.0, respectively. Recalculation of the affinity constants based on the concentration of glutamic acid in the solution yield  $K_T$ s of  $1.8 \pm 0.5 \mu\text{M}$  independent of the external pH, indicating that the protonated form of glutamate, i.e., glutamic acid, and glutamine are the transported species. The maximal rates of glutamate and glutamine uptake are independent of the extracellular pH as long as the intracellular pH is kept constant, despite large differences in the magnitude and composition of the components of the proton motive force. Uptake of glutamate and glutamine requires the synthesis of ATP either from glycolysis or from arginine metabolism and appears to be essentially unidirectional. Cells are able to maintain glutamate concentration gradients exceeding  $4 \times 10^3$  for several hours even in the absence of metabolic energy. The  $t_{1/2}$ s of glutamate efflux are 2, 12, and  $>30$  h at pH 5.0, 6.0, and 7.0, respectively. After the addition of lactose as energy source, the rate of glutamine uptake and the level of ATP are both very sensitive to arsenate. When the intracellular pH is kept constant, both parameters decrease approximately in parallel (between 0.2 and 1.0 mM ATP) with increasing concentrations of the inhibitor. These results suggest that the accumulation of glutamate and glutamine is energized by ATP or an equivalent energy-rich phosphorylated intermediate and not by the proton motive force.

With a few exceptions, streptococci are nutritionally fastidious, which means that many solutes have to be translocated from the medium into the cytoplasm. Detailed information is available about the regulation and energy coupling of sugar transport systems in streptococci (*Streptococcus cremoris*, *S. lactis*, and *S. diacetylactis*) (21). Studies on amino acid transport (1, 11, 20, 27) and ion movements (3, 10, 13) have been performed in *S. faecalis* and more recently in *S. cremoris* (8, 18). In *S. faecalis* the operation of two kinetically distinct dicarboxylic acid transport systems has been shown with affinities for glutamate and aspartate (20). Transport of aspartate and glutamate by the high-affinity system appears to be essentially unidirectional and requires ATP but not the proton motive force (11). The transport system for  $\alpha$ -aminoisobutyric acid, a nonmetabolizable analog of alanine, and a number of structurally similar neutral amino acids such as serine, alanine, and glycine has been studied in *S. lactis* ML3; this system is most likely driven by the proton motive force (26). The proton motive force has also been shown to be the driving force for the accumulation of (neutral) amino acids in *S. faecalis* (1).

The development of a method to incorporate proton pumps functionally into membrane vesicles of *S. cremoris* and other strictly fermentative bacteria by membrane fusion has facilitated the study of secondary transport systems in these organisms (7, 8). In fused membranes of *S. cremoris* the kinetics, energetics, and regulation of transport of ali-

phatic amino acids have recently been characterized (18a).

Recent studies with lactose-starved *S. cremoris* Wg2 have shown that upon exhaustion of the energy source the efflux rates for different amino acids vary considerably (19). Most amino acids equilibrate with the external medium at a rate determined by the dissipation of the proton motive force. In contrast, glutamate and aspartate leaked out from the cell very slowly. Clearly, the organism is able to prevent rapid loss of these quantitatively important amino acids under conditions of energy starvation. In growing *S. cremoris* and other streptococci, glutamate and aspartate constitute more than 70% of the total free amino acid pool (9; unpublished data).

These observations together with the evidence for ATP-driven transport systems for glutamate and aspartate in *S. faecalis* prompted us to investigate the transport of these amino acids in *S. lactis* ML3 and *S. cremoris* Wg2 in more detail. In this paper we report that glutamate and glutamine are taken up by a single, kinetically distinguishable transport system with a very low affinity for aspartate. The affinity of the transport system for glutamate depends on the protonated state of this amino acid. The data indicate that glutamic acid but not glutamate anion is taken up with an apparent affinity constant similar to that of glutamine. The maximal rates of glutamate and glutamine transport are independent of the magnitude and composition of the proton motive force but require the presence of ATP intracellularly.

Glutamate is defined herein as the sum of glutamic acid and glutamate anion present in the solution. Although

\* Corresponding author.

glutamic acid appears to be the transported species in *S. lactis* and *S. cremoris*, transport of this amino acid will be referred to as transport of glutamate.

## MATERIALS AND METHODS

**Culture conditions.** *S. cremoris* Wg2 and *S. lactis* ML3 were maintained in 10% (wt/vol) skimmed milk containing 0.1% (wt/vol) Trypton (Difco Laboratories) and stored at  $-20^{\circ}\text{C}$ . Precultures, made by transferring the organisms from the milk cultures to a complex medium (MRS) (5) at pH 6.4 containing 1.0% (wt/vol) lactose or galactose as the energy source, were grown overnight at  $30^{\circ}\text{C}$ . To induce arginine metabolism in *S. lactis* ML3, the cells were grown on galactose and 10 mM arginine (4).

**Transport assays.** Cells were harvested by centrifugation, washed two times, and suspended in buffer at 1 to 2 mg of protein per ml. To de-energize the cells, suspensions were incubated for 30 min at room temperature with 1 mM methyl-1-thio- $\beta$ -D-galactopyranoside. The cells were then washed twice and suspended as described in the figure legends or below. Transport was routinely assayed at  $30^{\circ}\text{C}$  in 200  $\mu\text{l}$  of incubation mixture, unless indicated otherwise. Initial uptake rates were determined by adding the appropriate concentration of  $^{14}\text{C}$ -labeled amino acid to the cell suspension after 3 to 5 min of preenergization with either 10 mM lactose or 5 mM arginine. After 5 to 30 s, the uptake reaction was stopped by the addition of 2 ml of ice-cold 100 mM LiCl, filtered on a 0.45- $\mu\text{m}$  cellulose nitrate filter (Millipore Corp.), and washed once more with 2 ml of ice-cold LiCl. For simultaneous measurements of transport activity, membrane potential, and pH gradient (see below), samples of 100  $\mu\text{l}$  were rapidly taken from the incubation mixture (2.0 ml) and diluted with LiCl as described above. Efflux and exchange activity were measured after preloading the cells (2 to 3 mg of protein per ml) with  $^{14}\text{C}$ -labeled glutamate (42  $\mu\text{M}$ ; 11.9 mCi/mmol) in the presence of 10 mM lactose for 15 min at  $30^{\circ}\text{C}$ . After treatment with methyl-1-thio- $\beta$ -D-galactopyranoside and two additional washing steps the cells were concentrated to 60 to 80 mg of protein per ml. Efflux and exchange were initiated after an 80-fold dilution of the cells as described below and in the figure legends. Filters were dried, and radioactivity was measured by liquid scintillation spectrometry.

**Extraction and identification of radioactive material.** Intracellular amino acids were identified by thin-layer chromatography. Uptake of amino acids was performed as described above, except that the cells were separated from the medium by silicone oil centrifugation (25). After neutralization of the perchloric acid extract, a portion of this solution was applied to silica gel plates. The amino acids were chromatographed with an *n*-butanol-acetic acid-water (4:1:1; vol/vol/vol) mixture (24). Amino acids were identified with ninhydrin reagent. Radioactivity was visualized by autoradiography. The radioactivity in the spots was quantitated by liquid scintillation spectrometry.

**Measurements of  $\Delta\Psi$  and  $\text{Z}\Delta\text{pH}$ .** For simultaneous measurements of transport activity, ATP concentration, membrane potential ( $\Delta\Psi$ ), and transmembrane proton gradient ( $\text{Z}\Delta\text{pH}$ ), cells were added to a polyvinylchloride vessel with a thermostat ( $30^{\circ}\text{C}$ ) in which an ion-selective tetraphenylphosphonium ion ( $\text{TPP}^+$ ) electrode and an ion-selective salicylate electrode (12) were inserted. The incubation mixture (2.0 ml) usually contained a cell suspension of 1 to 2 mg of protein per ml;  $\text{TPP}^+$  and potassium salicylate were added to 4 and 100  $\mu\text{M}$ , respectively. To measure the  $\text{Z}\Delta\text{pH}$

accurately, the distribution of salicylate was calculated from the change in electrode voltage upon the addition of 0.5  $\mu\text{M}$  nigericin. For uptake measurements and determination of the intracellular ATP concentration, samples (100  $\mu\text{l}$ ) were taken from the incubation mixture at the indicated time intervals and further handled as described in the figure legends. Alternatively, to measure pH gradients inside alkaline or inside acidic with respect to the external medium, the distribution of  $[\text{U-}^{14}\text{C}]\text{benzoic acid}$  (50 mCi/mmol) or  $[\text{U-}^{14}\text{C}]\text{methylamine}$  (56 mCi/mmol), respectively, was measured by the silicone oil centrifugation method (25).

**Calculations.** The  $\Delta\Psi$  was calculated by using the Nernst equation from the distribution of  $\text{TPP}^+$  between the bulk phase of the medium and the cytoplasm after correction for concentration-dependent binding of  $\text{TPP}^+$  to the cytoplasmic membrane (15). Binding of  $\text{TPP}^+$  was measured in cells deenergized by incubation with 1% (vol/vol) toluene for 1 h at  $37^{\circ}\text{C}$ . The observed binding constant ( $K$ ) for  $\text{TPP}^+$  varied between 8 and 15 in different experiments and was independent of the extracellular pH. For the calculation of the  $\Delta\Psi$  and  $\text{Z}\Delta\text{pH}$  a specific internal volume of 2.9 and 3.6  $\mu\text{l}/\text{mg}$  of protein was used for *S. lactis* and *S. cremoris*, respectively (19).

**Miscellaneous.** Extraction procedures for ATP analysis and measurement of the free intracellular amino acid pool have been described previously (19). ATP concentrations were determined with the firefly luciferase assay as described previously (17). Amino acids were analyzed after derivatization with dansylchloride and separated by reverse-phase high-performance liquid chromatography (19). Protein was measured by the method of Lowry et al. (16) with bovine serum albumin as a standard.

**Materials.**  $^3\text{H}_2\text{O}$  (1 mCi/ml),  $[\text{U-}^{14}\text{C}]\text{benzoate}$  (50 mCi/mmol),  $[\text{U-}^{14}\text{C}]\text{methylamine}$  (56 mCi/mmol),  $[\text{U-}^{14}\text{C}]\text{glutamate}$  (280 mCi/mmol),  $[\text{U-}^{14}\text{C}]\text{glutamine}$  (280 mCi/mmol), and  $[\text{U-}^{14}\text{C}]\text{leucine}$  (333 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, United Kingdom. All other chemicals were reagent grade and were obtained from commercial sources.

## RESULTS

**Substrate specificity of the transport system.** Examination of the initial rates of glutamate uptake by *S. lactis* ML3 and *S. cremoris* Wg2 over a wide range of concentrations (1.8  $\mu\text{M}$  to 1.0 mM) showed saturation kinetics. Transformation of the experimental data into Eadie-Hofstee plots yielded single straight lines (Fig. 1A), indicating the presence of only one kinetically distinguishable glutamate transport system. The  $K_{\text{T}}$ s of glutamate uptake at pH 6.0 were 77 and 36  $\mu\text{M}$  for *S. lactis* and *S. cremoris*, respectively. Surprisingly, glutamate uptake was competitively inhibited by glutamine. The  $K_i$  for glutamine inhibition was  $2.0 \pm 0.5 \mu\text{M}$  in both organisms, whereas aspartate inhibited with a  $K_i$  larger than 1 mM.  $\alpha$ -Ketoglutarate and oxaloacetate up to 1.0 mM had no inhibitory effect on glutamate transport in *S. cremoris* Wg2.

The  $K_{\text{T}}$ s for glutamine uptake in *S. lactis* and *S. cremoris* at pH 6.0 were 2.6 and 1.9  $\mu\text{M}$ , respectively. Glutamine transport in *S. lactis* cells was competitively inhibited by glutamate with a  $K_i$  of 68  $\mu\text{M}$  at pH 6.0, showing that the affinity constants for transport of these amino acids are comparable to the affinity constants for the inhibition.

Since arginine can be used as a source of energy for ATP synthesis in *S. lactis* but not in *S. cremoris* (4), the effect of arginine on the initial rate of glutamine uptake in *S. cremoris*

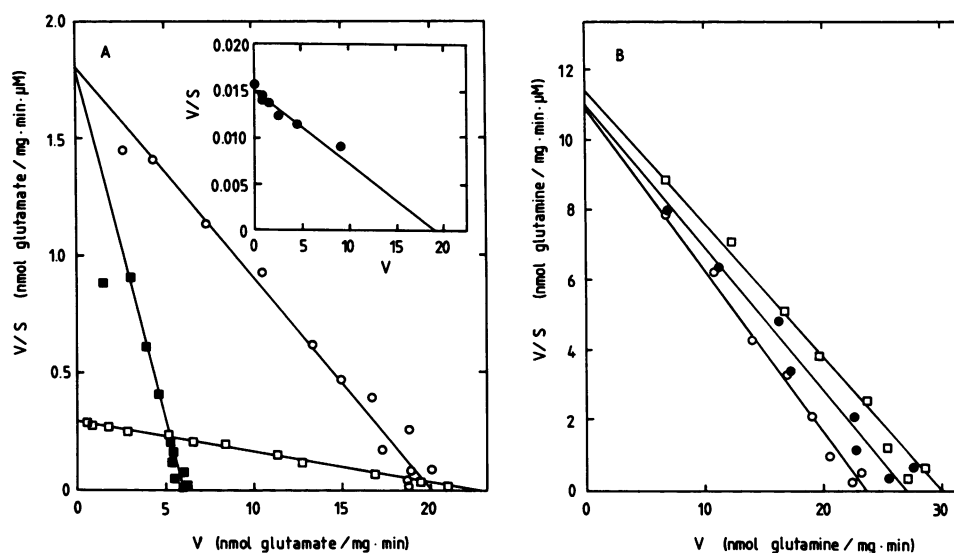


FIG. 1. Effect of external pH on the kinetic parameters of glutamate (A) and glutamine (B) transport in *S. lactis* ML3. A concentrated cell suspension in 10 mM K-PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)]–80 mM KCl–5 mM MgCl<sub>2</sub> at pH 6.0 was diluted to a final protein concentration of 0.4 mg/ml in a 30 mM K-MES (morpholine ethanesulfonic acid)–30 mM K-PIPES–30 mM K-HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid)–5 mM MgCl<sub>2</sub> buffer containing 10 mM lactose at pH 4.0 (■), 5.1 (○), 6.0 (□), and 7.0 (●). After 3 min of pre-energization, various concentrations (1.8  $\mu$ M to 1.03 mM) of [<sup>14</sup>C]glutamate (A) or [<sup>14</sup>C]glutamine (B) were added. Uptake was measured between 5 and 30 s, after which transport was terminated as described in Materials and Methods. Initial rates of uptake were measured in duplicate; the data are plotted in the form of Eadie-Hofstee plots (6). The data for the initial rates of glutamate uptake at pH 7.0 are shown on a different scale in the inset of panel A.

was measured. At pH 6.0 glutamine transport in glycolyzing cells of *S. cremoris* appeared to be competitively inhibited by arginine with a  $K_i$  of 257  $\mu$ M.

**Effect of the extracellular pH on the kinetic properties of glutamate and glutamine transport.** Kinetic analysis of glutamate transport in *S. lactis* ML3 revealed that the maximal rate of glutamate uptake ( $V_{\max}$ ) is independent of the external pH between pH 5 and 7, whereas the apparent affinity ( $K_T$ ) of the transport system increases dramatically with increasing pH (Fig. 1A). The  $K_T$ s for glutamate uptake were 11.2, 77, and 1,200  $\mu$ M at pH 5.1, 6.0, and 7.0, respectively. A further increase in the affinity for glutamate was observed upon lowering the pH to 4.0 (Fig. 1A). Glutamate has  $pK_a$  values of 2.19, 4.25, and 9.67 and the relative concentrations of the different species of glutamate between pH 4.0 and 7.0 are mainly determined by the protonation state of the  $\gamma$ -carboxyl group. According to the Henderson-Hasselbach equation the ratios of glutamic acid to the glutamate anion are 1.778, 0.141, 0.018, and 0.002 at pH 4.0, 5.1, 6.0, and 7.0, respectively. From these data the concentrations of glutamic acid and the glutamate anion can be calculated at a given pH. Replotting of the initial rates of uptake at various external pHs, on the assumption that only glutamic acid can be transported, shows very similar  $K_T$ s ( $1.8 \pm 0.5 \mu$ M) between pH 4.0 and 7.0 (Fig. 2). Under identical conditions the affinity for glutamate was independent of the extracellular pH with  $K_T$ s of  $2.5 \pm 0.3 \mu$ M (Fig. 1B). This indicates that the affinity of the transport system does not change significantly in the pH range investigated.

**Effect of the extracellular pH on the magnitude and composition of the proton motive force and the intracellular ATP concentration.** To elucidate the mechanism of energy coupling to glutamate and glutamine transport, the magnitude of the components of the proton motive force and the ATP levels were measured between pH 5.1 and 7.8 (Fig. 3). Steady-state values of the  $\Delta\Psi$  and the  $\Delta pH$  were deter-

mined 5 min after the addition of lactose to the cell suspension. At pH 5.1 the proton motive force of *S. lactis* cells was largely composed of a pH gradient. With increasing external pH, the  $\Delta pH$  decreased as the intracellular pH remained fairly constant, i.e., between 7.2 and 7.4. The decrease in  $\Delta pH$  was only partially compensated by an increase in the  $\Delta\Psi$ . Consequently the proton motive force decreased from  $-135$  mV at pH 5.1 to  $-60$  mV at pH 7.4. The intracellular ATP concentration was maximal at pH 5.8 (i.e., 2.5 mM) and decreased to 1.5 mM as the extracellular pH was raised to pH 7.4. In the presence of nigericin, the ATP content of the cells was maximal above pH 7.0 and decreased with decreasing pH. The addition of nigericin made the interior of the cell about 0.7 pH units more acidic than the external medium between pH 5 to 8 (data not shown).

The independence of the  $V_{\max}$  of glutamate and glutamine uptake of the external pH (between pH 5.1 and 7.0) (Fig. 1A and B, Fig. 2), despite dramatic changes in the magnitude of the  $\Delta\Psi$  and the  $\Delta pH$  (Fig. 3), indicates that the transport system is not simply driven by the proton motive force. In accordance with this are the observations that imposition of an artificial membrane potential (potassium diffusion potential, inside negative) or a pH gradient (acetate diffusion potential or pH jump, inside alkaline) or both (W. N. Konings, W. De Vrij, A. J. M. Driessen, and B. Poolman, in *Sugar Transport and Metabolism in Gram-Positive Bacteria*, in press) failed to induce glutamate uptake in starved cells of *S. lactis* ML3 and *S. cremoris* Wg2. Control experiments in which leucine uptake was monitored indicated that the methods employed resulted in the transient generation of a  $\Delta\Psi$  or  $\Delta pH$  (or both) of about  $-100$  mV (data not shown).

**Identification of accumulated radioactive material.** Cells of *S. lactis* ML3 were permitted to accumulate <sup>14</sup>C-labeled glutamate and glutamine for 5 min. Extraction, chromatography, and autoradiography of the accumulated material showed that a small fraction (9%) of glutamate was con-

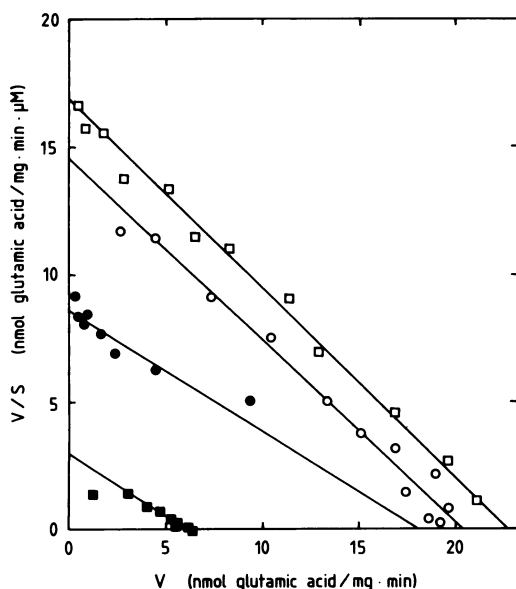


FIG. 2. Effect of external pH on the kinetic parameters of the glutamate transport system in *S. lactis* ML3 on the assumption that glutamic acid is the transported substrate. Experimental data were taken from the experiment shown in Fig. 1A. The concentrations of glutamic acid were calculated with the Henderson-Hasselbach equation, assuming that the charge of the dicarboxylic acid is determined by the protonation state of the  $\gamma$ -carboxyl group ( $pK_a$  of 4.25) in the pH range investigated. Symbols are as shown in the legend to Fig. 1A.

verted into glutamine. Glutamine, on the other hand, was largely (94%) converted into glutamate after 5 min of incubation. No unidentified products were observed, even after longer periods of incubation. To establish whether the conversion of glutamine into glutamate affected the transport rate of glutamine, cells were preincubated for 5 min with the inhibitors of glutamine metabolism: azaserine or 6-diazo-5-oxo-L-norleucine. In the presence of azaserine a smaller but still significant amount of glutamine was converted into glutamate, but in the presence of 6-diazo-5-oxo-L-norleucine glutamine remained essentially unchanged. These inhibitors of glutamine metabolism had no effect on the initial rate of glutamine transport (data not shown).

**Unidirectional nature of the glutamate-glutamine transport system.** The intracellular free glutamine pool of methyl-1-thio- $\beta$ -D-galactopyranoside-treated *S. lactis* ML3 cells was 19.0 mM. These cells accumulated [ $^{14}$ C]glutamate to various levels, depending on the extracellular glutamate concentration (Table 1). Cells metabolizing lactose took up nearly 100% of the glutamate when the initial concentration was below 15  $\mu$ M and at a protein concentration of 0.22 mg/ml. The established concentration gradients for glutamic acid (the transported species), taking into account the pH difference across the cytoplasmic membrane, exceeded  $4 \times 10^3$  and could largely be maintained for several hours even in the absence of metabolic energy. When the extracellular glutamate concentration was increased the intracellular concentration appeared to be restricted to a maximum of about 80 mM, corresponding with a glutamic acid concentration of 90  $\mu$ M (Table 1).

Transport of glutamate and glutamine in *S. lactis* could also be driven by arginine metabolism (data not shown). The initial rates of uptake in the presence of arginine were always lower than with lactose as energy source for ATP synthesis,

due to the (competitive) inhibition of glutamate and glutamine transport by high concentrations of arginine (see above).

Exit of glutamate from methyl-1-thio- $\beta$ -D-galactopyranoside-treated cells was studied after preloading with [ $^{14}$ C]-labeled glutamate. The pH dependence of glutamate efflux from energy-starved *S. lactis* cells in the presence of valinomycin and nigericin is shown in Fig. 4. The rate of glutamate efflux increases with decreasing pH, but the  $t_{1/2}$ s for efflux are 2 to 3 orders of magnitude larger than for amino acids which are facilitated by secondary transport mechanisms (i.e., alanine, glycine, serine, leucine, and others) (8a). The  $t_{1/2}$ s of glutamate efflux were 125 min, 12 h, and >30 h at pH 5.0, 6.0, and 7.0, respectively. The intracellular glutamic acid concentrations are 2,946, 341 and 35  $\mu$ M at pH 5.0, 6.0, and 7.0, respectively. The glutamic and concentration gradients are equal at these pH values because internal and external glutamic acid concentrations are influenced in the same manner by a change in medium pH. It is also unlikely that the efflux rates at various pHs have been affected by kinetic limitation of the transport system, since exit of [ $^{14}$ C]glutamate in the presence of 10 mM unlabeled glutamate (exchange) was nearly independent of the pH (data not shown). The  $t_{1/2}$ s of exchange varied between 65 to 85 min, corresponding to a glutamate exit rate of about 0.2 nmol/min per mg of protein on the basis of a total intracellular glutamate concentration of 19.5 mM.

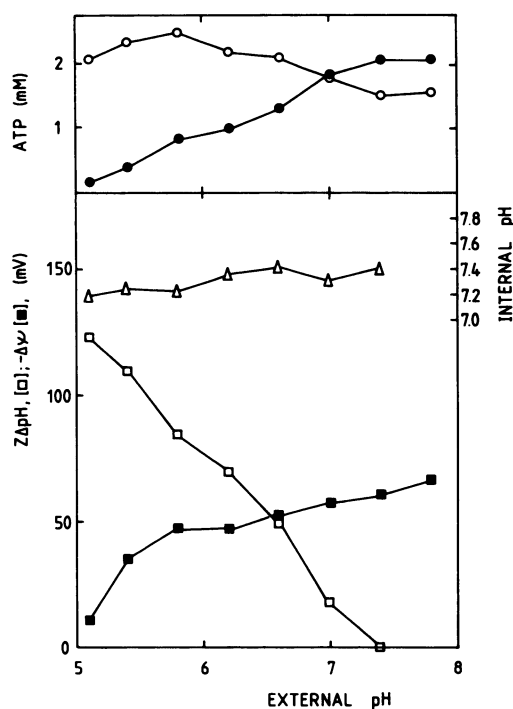


FIG. 3. Effect of external pH on the components of the proton motive force, the internal pH, and the intracellular ATP concentration in *S. lactis* ML3. Cells were suspended in 30 mM K-MES-30 mM K-PIPES-30 mM K-HEPES-5 mM  $MgCl_2$  at the indicated pH at 30°C to a final protein concentration of 2.1 mg/ml. The  $\Delta\Psi$  (■) and the  $Z\Delta pH$  (□) were determined from the distribution of TPP $^+$  and salicylate, respectively, by using ion-selective electrodes. Samples (100  $\mu$ l) were withdrawn for the determination of the intracellular ATP concentration 5 min after the addition of 10 mM lactose. Other symbols: internal ATP concentrations in the absence (○) and presence (●) of 0.5  $\mu$ M nigericin, internal pH ( $\Delta$ ).

**Glutamine uptake as a function of the intracellular ATP levels.** Since glutamate and glutamine transport in *S. lactis* appears to rely upon ATP either from glycolysis or arginine catabolism, the effect of the intracellular ATP concentration on transport was studied. Cells were allowed to glycolyze at pH 7.5 in the presence of various concentrations of arsenate. Under these conditions the intra- and extracellular pHs are approximately the same (Fig. 3; see below). ATP synthesis in glycolyzing cells of *S. lactis* ML3 is effectively inhibited by arsenate (Fig. 5). Arsenate at a concentration of 10  $\mu$ M was sufficient to lower the ATP levels by more than 60%. A further increase in arsenate concentrations (up to 2 mM) resulted in a maximal decrease in ATP by 80 to 85%. The initial rate of glutamine transport was not significantly inhibited by a decrease in ATP level from 1.27 to 0.9 mM, but fell to 1 to 2% of the maximal activity upon a further decrease to 0.2 to 0.3 mM. The intracellular pHs were 7.46, 7.46, 7.35, and 7.27 in glycolyzing cells in the presence of 0, 2, 8, and 40  $\mu$ M arsenate, respectively. The relationship between the rate of glutamine uptake and the intracellular ATP concentration is plotted in the inset of Fig. 5. Strikingly, *S. lactis* cells which derived their ATP from arginine metabolism via the arginine dihydrolase pathway (4) were insensitive toward arsenate. Transport of glutamine was also not markedly affected by arsenate in these cells (data not shown).

### DISCUSSION

The glutamate-glutamine transport system described in this paper differs from most amino acid carriers described thus far in several aspects. First, the substrate specificity is unusual, since the transport system catalyzes the translocation of a neutral amino acid and one that is predominantly negatively charged at physiological pH values. The pH dependence of the  $K_T$  for glutamate transport and the pH independence of the  $K_T$  when it is assumed that glutamic acid is the translocated substrate indicates that only the neutral species is transported with a  $K_T$  of about 2  $\mu$ M. The independence of the  $K_T$  of glutamine transport on the external pH shows that the uptake has not been affected by a protonation of an essential ionizable group of the transport

TABLE 1. Effect of external glutamic acid on the glutamic acid concentration gradients of *S. lactis* ML3<sup>a</sup>

Initial external glutamate concn <sup>b</sup> ( $\mu$ M)	Glutamic acid concn ( $\mu$ M)			Concn gradient <sup>f</sup>
	Initial external <sup>c</sup>	Final external <sup>d</sup>	Final internal <sup>e</sup>	
10	0.175	0.012	53.1	4,422
30	0.526	0.184	88.1	479
100	1.754	1.402	90.0	64

<sup>a</sup> Cells were washed and suspended in buffer containing 50 mM potassium-morpholineethanesulfonic acid and 5 mM MgSO<sub>4</sub> (pH 6.0). Uptake was initiated by diluting the cells to a final protein concentration of 0.22 mg/ml into the same buffer (2.0 ml, final volume) containing 10 mM lactose and various initial [<sup>14</sup>C] glutamate concentrations. Samples (0.1 ml) were withdrawn periodically, and the cells were filtered and washed as described in Materials and Methods. The steady-state internal and external glutamate concentrations were determined from the amount of [<sup>14</sup>C] glutamate taken up by the cells and the preexisting free glutamate pool (19.0 mM) in the starved cells.

<sup>b</sup> Initial concentration of glutamate in the transport assay.

<sup>c</sup> Calculated initial glutamic acid concentration at pH 6.0.

<sup>d</sup> Final glutamic acid concentration, calculated from the external concentration of glutamate in the steady state.

<sup>e</sup> Internal glutamic acid concentration in the steady state, calculated from the total amount of intracellular glutamate on the basis of an internal pH of 7.2 (Fig. 3).

<sup>f</sup> Concentration gradients were calculated from the total intracellular glutamic acid concentration divided by the final external glutamic acid concentration in the transport assay.

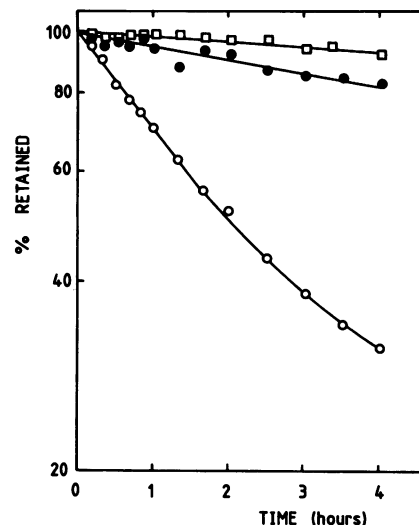


FIG. 4. pH dependence of glutamate efflux from *S. lactis* ML3 cells. [<sup>14</sup>C]glutamate-loaded cells (57.6 mg of protein per ml) were diluted 80-fold into 50 mM potassium phosphate–5 mM MgSO<sub>4</sub> buffer (2.0 ml, final volume) of the appropriate pH containing 1.0  $\mu$ M nigericin and 2.0  $\mu$ M valinomycin. Samples (0.1 ml) were taken at the times indicated and further treated as described above. The pHs were 5.0 (○), 6.0 (●), and 7.0 (□). The total intracellular glutamate pool at zero time was 19.5 mM.

system. Evidence that transport proteins can only bind the neutral species of a carboxylic substrate is scarce in the literature. In some cases, such as succinate uptake by membrane vesicles of *Escherichia coli* (22), the  $K_T$  for transport increases with increasing external pH. A distinc-

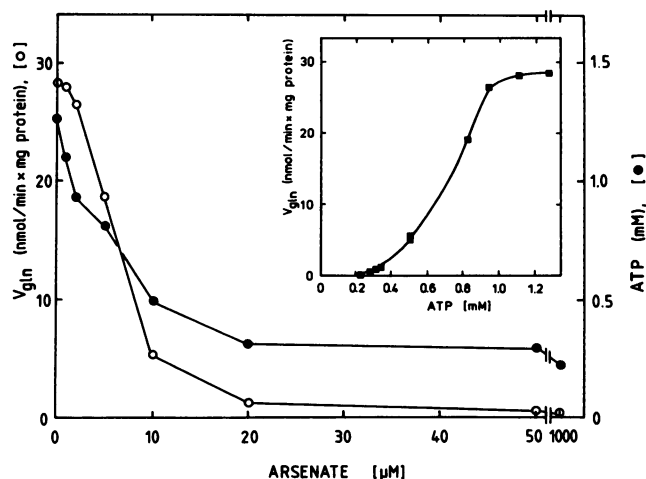


FIG. 5. Effect of arsenate on the initial rate of glutamate uptake and the ATP levels of *S. lactis* ML3. Cells were suspended to a final protein concentration of 0.75 mg/ml in 50 mM K-PIPES–5 mM MgSO<sub>4</sub> buffer (pH 7.5) containing 10 mM lactose and various concentrations of arsenate (sodium salt). After 5 min of glycolysis, cell metabolism was quenched by the addition of perchloric acid (5% [wt/wt], final concentration) and the extracts were further handled for the analysis of ATP as described previously (19). The initial rate of glutamine uptake was measured in parallel samples upon the addition of 10.9  $\mu$ M [<sup>14</sup>C]glutamine after 5 min of energization. The initial rate of glutamine uptake (○) and the intracellular ATP concentrations (●) are plotted. The dependence of the glutamine uptake rate on the internal ATP levels is shown in the inset.

tion between the acidic or the (di)anionic species as translocated substrate is difficult to make since protons are symported with succinate in *E. coli*. Furthermore, an ordered transport mechanism in which protons bind first may facilitate the binding (i.e., increase the affinity) of the secondary solute. Glutamate uptake in *S. faecalis* is mediated by a high-affinity system ( $K_T$  of 30  $\mu$ M) and a low-affinity transport system ( $K_T$  of 12 mM) (20). Although glutamate transport by the low-affinity system is inhibited by glutamine, this transport system does not resemble the one described in this paper since the initial rate of glutamate uptake was independent of the presence of an energy source (27). This activity most likely represents exchange of labeled extracellular amino acid with a preexisting intracellular glutamate pool. The high-affinity glutamate transport system is competitively inhibited by aspartate ( $K_i$  of 7  $\mu$ M) (20). A similar substrate specificity is observed for the glutamate transport systems of *E. coli* (23) and *Bacillus subtilis* (14). The lack of inhibition of glutamate transport in *S. cremoris* by  $\alpha$ -ketoglutarate shows that the amino group of the  $\alpha$ -carbon is essential for binding to the carrier.

The proton motive force is not involved as a driving force for glutamate-glutamine transport, as is shown directly by the independence of the initial rate of glutamate and glutamine uptake of the external pH, despite large variations in the magnitude of the  $\Delta\text{pH}$  and the  $\Delta\Psi$  (Fig. 3). *S. lactis* and *S. cremoris* can accumulate glutamic acid to a concentration gradient of  $4 \times 10^3$  (Table 1), which exceeds the thermodynamic limits set by the proton motive force (assuming that one  $\text{H}^+$  ion accompanies the translocation of one glutamic acid molecule). Moreover, artificial ion gradients failed to induce the concentrative uptake of glutamate or glutamine. Finally, the essential irreversibility of glutamate transport is not in accordance with the reversible secondary transport processes (Konings et al., in press).

The inability of *S. lactis* cells to maintain the intracellular pH constant below an external pH of 5.0 is most likely the major cause for the decreased rate of glutamate uptake at pH 4.0. The aspect of regulation by the intracellular pH and the effects of uncouplers and ionophores on glutamate and glutamine uptake is described elsewhere (18a).

Uptake of glutamate and glutamine is supported by both glycolysis and arginine catabolism and therefore probably requires ATP or an other energy-rich phosphate bond intermediate. This conclusion is supported by the observed correlation between the rate of glutamine transport and the intracellular ATP pool. The data suggest that the transport activity is maximal at ATP concentrations above 0.9 mM (Fig. 5, inset). The low rate of glutamate and glutamine uptake in the presence of arginine as the energy source for ATP synthesis has several causes. The effect is partly explained by the lower ATP content of the cell in the presence of arginine (about 0.8 mM, unpublished results) in comparison to that in cells metabolizing lactose (Fig. 5). The lower intracellular pH in the presence of arginine will also affect transport (18a). Most pronounced, however, is the (competitive) inhibition of glutamate and glutamine transport by millimolar concentrations of arginine. In *S. cremoris*, which cannot utilize arginine as an energy source, glutamine transport is inhibited by arginine with a  $K_i$  of 257  $\mu$ M.

The relative insensitivity of ATP synthesis in arginine catabolism to arsenate may be caused by the failure of the cells to accumulate arsenate in the presence of arginine, as has been shown for *S. faecalis* (11). On the other hand, ornithine transcarbamylase, which catalyzes the conversion of L-citrulline and phosphate into L-ornithine and carbamoyl

phosphate, may be less sensitive to metabolic uncoupling by arsenate than glyceraldehyde 3-phosphate dehydrogenase.

The energetics of glutamate and glutamine transport in *S. lactis* ML3 and *S. cremoris* Wg2 is consistent with the observations made for arsenate (phosphate) and aspartate transport in *S. faecalis* (11). Transport of these solutes has also been proposed to be energized by ATP or an energy-rich phosphorylated intermediate. The glutamate-glutamine transport system of *S. lactis* differs from the aspartate-glutamate transport system of *S. faecalis* with respect to its kinetics and regulation by the intracellular pH (11, 18a, 20, 27).

The scanty information on ATP-dependent transport systems in gram-positive organisms has recently been summarized (Konings et al., in press). With a few exceptions, all of these systems translocate inorganic cations by mechanisms in which ATP may provide directly the energy for solute translocation or fulfills a role in regulating transport. The same applies for the binding protein-dependent transport systems in gram-negative bacteria. A role for the membrane potential is sometimes implicated in either the energization or the regulation of ATP-dependent transport (2). The experiments presented in this paper suggest that phosphate bond energy drives the accumulation of glutamate and glutamine in *S. lactis* and *S. cremoris* without the involvement of the proton motive force.

#### ACKNOWLEDGMENTS

We thank A. J. M. Driessen and K. J. Hellingwerf for valuable suggestions throughout this work.

These investigations were supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

#### LITERATURE CITED

1. Asghar, S. S., E. Levin, and F. M. Harold. 1973. Accumulation of neutral amino acids by *Streptococcus faecalis*. J. Biol. Chem. **248**:5225-5233.
2. Bakker, E. P., and F. M. Harold. 1980. Energy coupling to potassium transport in *Streptococcus faecalis*. Interplay of ATP and the proton motive force. J. Biol. Chem. **255**:433-440.
3. Booth, I. R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49**:359-378.
4. Crow, V. L., and T. D. Thomas. 1982. Arginine metabolism in lactic streptococci. J. Bacteriol. **150**:1024-1032.
5. De Man, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130-135.
6. Dixon, M., and E. C. Webb. 1979. Enzymes, 3rd ed. Longman Group Ltd., London.
7. Driessen, A. J. M., K. J. Hellingwerf, W. N. Konings. 1985. Light-induced generation of a proton motive force and  $\text{Ca}^{2+}$ -transport in membrane vesicles of *Streptococcus cremoris* fused with bacteriorhodopsin proteoliposomes. Biochim. Biophys. Acta **808**:1-12.
8. Driessen, A. J. M., W. De Vrij, W. N. Konings. 1985. Incorporation of beef heart cytochrome c oxidase as a proton-motive force-generating mechanism in bacterial membrane vesicles. Proc. Natl. Acad. Sci. USA **82**:7555-7559.
- 8a. Driessen, A. J. M., J. Kodde, S. de Jong, and W. N. Konings. 1987. Neutral amino acid transport by membrane vesicles of *Streptococcus cremoris* is subject to regulation by internal pH. J. Bacteriol. **169**:2748-2754.
9. Griffith, C. J., and T. H. Melville. 1974. Influence of growth conditions on the composition of some streptococcal amino acid pools. Microbios **9**:7-13.
10. Harold, F. M., and Y. Kakinuma. 1985. Primary and secondary transport of cations in bacteria. Ann. N.Y. Acad. Sci. **456**:375-383.

11. Harold, F. M., and E. Spitz. 1975. Accumulation of arsenate, phosphate, and aspartate by *Streptococcus faecalis*. J. Bacteriol. 122:266-277.
12. Hellingwerf, K. J., and P. Van Hoorn. 1985. A polyvinylchloride-membrane based anion selective electrode for continuous registration of  $\Delta\text{pH}$  (interior alkaline) with salicylate as the indicator probe. J. Biochem. Biophys. Methods 11:83-93.
13. Kobayashi, H., J. Van Brunt, and F. M. Harold. 1978. ATP-linked calcium transport in cells and membrane vesicles of *Streptococcus faecalis*. J. Biol. Chem. 253:2085-2092.
14. Konings, W. N., A. Bisschop, and M. C. C. Daatselaar. 1972. Transport of L-glutamate and L-aspartate by membrane vesicles of *Bacillus subtilis* W23. FEBS Lett. 24:260-264.
15. Lolkema, J. S., K. J. Hellingwerf, and W. N. Konings. 1982. The effect of "probe-binding" on the quantitative determination of the proton-motive force in bacteria. Biochim. Biophys. Acta 681:85-94.
16. Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
17. Otto, R., B. Klont, B. ten Brink, and W. N. Konings. 1984. The phosphate potential, adenylate energy charge and proton motive force in growing cells of *Streptococcus cremoris*. Arch. Microbiol. 139:338-343.
18. Otto, R., R. G. Lageveen, H. Veldkamp, and W. N. Konings. 1982. Lactate-efflux induced electrical potential in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. 149:733-738.
- 18a. Poolman, B., K. J. Hellingwerf, and W. N. Konings. 1987. Regulation of the glutamate-glutamine transport system by intracellular pH in *Streptococcus lactis*. J. Bacteriol. 169:2272-2276.
19. Poolman, B., E. J. Smid, H. Veldkamp, and W. N. Konings. 1986. Bioenergetic consequences of lactose starvation for continuously cultured *Streptococcus cremoris*. J. Bacteriol. 169:1460-1468.
20. Reid, K. G., N. N. Utech, and J. T. Holden. 1970. Multiple transport components for dicarboxylic amino acids in *Streptococcus faecalis*. J. Biol. Chem. 245:5261-5272.
21. Reizer, J., J. Deutscher, S. Sutrina, J. Thompson, and M. H. Saier. 1985. Sugar accumulation in gram-positive bacteria: exclusion and expulsion mechanisms. Trends Biochem. Sci. 10:32-35.
22. Robertson, D. E., G. J. Kaczorowski, M. L. Garcia, and H. R. Kaback. 1980. Active transport in membrane vesicles from *Escherichia coli*: the electrochemical proton gradient alters the distribution of the lac carrier between two different kinetic states. Biochemistry 19:5692-5702.
23. Schellenberg, G. D., and C. E. Furlong. 1977. Resolution of the multiplicity of the glutamate and aspartate transport systems of *Escherichia coli*. J. Biol. Chem. 252:9055-9064.
24. Stahl, E. 1962. Dunnschicht-chromatographie: ein laboratoriumshandbuch. Springer-Verlag, Berlin.
25. Ten Brink, B., and W. N. Konings. 1982. The electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* grown in batch culture. J. Bacteriol. 152:682-686.
26. Thompson, J. 1976. Characteristics and energy requirements of an  $\alpha$ -aminoisobutyric acid transport system in *Streptococcus lactis*. J. Bacteriol. 127:719-730.
27. Utech, N. M., K. G. Reid, and J. T. Holden. 1970. Properties of a dicarboxylic amino acid transport-deficient mutant of *Streptococcus faecalis*. J. Biol. Chem. 245:5273-5280.